PATENT

Attorney Docket No.: 6056-272RE

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re:	Patent application of E. Premkumar Reddy, et al.	:	Group Art Unit: 1626
Serial No.:	10/630,397	:	
Filed:	July 29, 2003	:	Examiner: Fiona Powers
For:	1-(4-SULFAMYLARYL)-3-SUBSTITUTED-5-ARYL-2-PYRAZOLINES AND INHIBITORS OF CYCLOOXYGENASE-2	:	

DECLARATION UNDER 37 C.F.R. § 1.132

Mail Stop Amendment
Commissioner for Patents
P.O. Box 1450
Alexandria. VA 22313-1450

1. I, M. V. Ramana Reddy, Ph.D., am one of the inventors named in the above-identified patent application. I am a member of the faculty of the Fels Institute for Cancer Research in the Temple University School of Medicine, Philadelphia, Pennsylvania.

CERTIFICATE OF MAILING
UNDER 37 C.F.R. 1.8(a)

I hereby certify that this paper, along with any paper referred to as being attached or enclosed, is being deposited with the United States Postal Service on the date indicated below, with sufficient postage, as first class mail, in an envelope addressed to: Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.

BY Jeannette Rayfield

DATE: January 19, 2006

2. The experimentation described in this declaration was conducted by me, in whole or in part, or under my supervision.

3. Attached as Exhibit 2 is a list assigning code numbers (eg., "ON xxxxx") is provided for ease of reference to the compound 1-(4-sulfamylphenyl)-3-trifluoromethyl-5-(3-indolyl)-2-pyrazoline [ON09250], and certain analogs thereof containing substituents on the indolyl moiety. ON09250 inhibited the growth of HT29 colon cancer cells in soft agar in an assay similar to the "Soft Agar Assay" set forth in the present patent application. HT29 cells were plated in 4% noble agar containing ON09250. Colony formation occurred over a period of 20 days, at which time the plates were stained with a 0.05% (w/v) nitroblue tetrazolium solution for 48 hours to stain for viable cells. Results of this experiment are shown in attached Exhibit 3. The results demonstrate that even at the lower concentration tested (15μM), ON09250 completely inhibited anchorage-independent growth of colorectal carcinoma cells. In this study, ON09250 demonstrated a much higher inhibitory activity than did celecoxib, another COX-2 inhibitor. An examination of the soft agar plates revealed that the plates of colorectal tumor cells treated with celecoxib contained many viable cells which never formed microscopic colonies. This indicates that the inhibition of colony growth with celecoxib was not due to cell death, but rather, to the cessation of cell proliferation (see attached Exhibit 4). In sharp contrast, the ON09250-treated plates were devoid of all viable cells at the concentrations above 20 μM. These experiments demonstrate that ON09250 induces both growth arrest and death of HT29 cells.

4. I prepared the analogs of ON 09250 identified in Exhibit 2: ON 09270, ON 09280, ON 09290, ON 09300, ON 09310, and ON 09320. The analogs were tested for dose-dependent inhibition of HT29 human colon cancer cells. As illustrated in attached Exhibit 5, several of the compounds had anti-tumor cell activity greater than that observed for celecoxib, and greater than that previously observed for ON 09250. Cells (count: 1 $x10^5$) were plated into 6-well dishes and after 24 hours incubation, each compound was added at five different concentrations, ranging over a 2 log dilution (1 μM -100 μM). The total number of viable cells

was determined after 96 hours of continuous treatment by staining with trypan blue and counting the number of non-staining cells (viable) remaining in each well using a hemacytometer. The percentage of viable cells remaining was calculated as follows: number of viable cells (compound treated) / (number of viable cells (DMSO treated) * 100). Dose response curves were generated by plotting the percentage of cells at each concentration against concentration tested.

5. In another experiment, ON09250 and certain of its analogs were tested against various human cancer cell lines, including breast cancer (BT-20 and MCF-7), brain cancer (HB-126 and HB-231), colon cancer (DLD-1), and prostate cancer cells (DU-145 and LNCAP). The EC50 value for each compound with respect to each particular type of cell are shown in Exhibit 6. The compounds were, for the most part, more effective at killing than celecoxib. The EC50 values for the test compounds were typically several-fold lower than the EC50 values obtained for celecoxib. Cytotoxicity assays were conducted as above in section 4.

6. A quantitative cell killing assay was conducted using HCT15 colon cancer cells. HCT15 cells express a significant amount of COX-2 enzyme and are known to be sensitive to NSAIDs. As shown in Exhibit 7, ON 09250 and celecoxib were equally effective in this assay with an EC50 value of approximately 25 μM. In the same assay, compound ON 09310 was found to be even more potent with an EC50 of between 1-25 μM (see attached Exhibit 8). Cytotoxicity assays were conducted as above in section 4.

7. Compound ON 09250 was tested for apoptosis induction in human tumor cell lines. As shown in Exhibit 9, ON 09250 induces apoptosis in HT29 human colon cancer cells and in MDA-MB-231 human breast cancer cells. Celecoxib, in contrast, did not induce apoptosis in the same cells. Apoptotic death of cells following treatment of ON 09250 was verified by Tunel assays. HT29 and MDA-MB-231 cells were treated with 20 μM ON 09250 or 40 μM celecoxib for 48 hours. Cells were harvested and washed one time with ice cold phosphate buffered with saline (PBS). The cells were cytospun, fixed in 4% paraformaldehyde

for 30 minutes, washed with PBS and permeabilized in 0.1% sodium citrate. The cells were then washed in PBS and treated with a labeling mixture that contained flourescein-labeled dUTP. The labeled cells were washed, mounted, and analyzed using confocal microscopy. Only the cells treated with ON 09250 were end-labeled with fluroscein-labeled dUTP by the terminal transferases. Green labeled cells also show the distinctive signs of chromatin fragmentation. The cells were counter-stained with propidium iodide (PI, red label) to verify the presence of cells following treatment and labeling procedures. These results show that colorectal carcinoma cells treated with ON 09250 undergo cell death by activating the apoptotic pathway, whereas celecoxib treatment inhibits the growth of these cells but does not induce apoptosis.

8. I also examined the effect of certain compounds on neuronal cell protection by way of COX-2 inhibition, assayed by evaluation of the inhibition of prostaglandin production. ON 09250, ON 09300, and ON 09310 were found to inhibit NMDA-induced neuronal prostaglandin production. These results were specific for COX-2 inhibition. This is because these compounds were also shown not to be direct NMDA receptor antagonists, as the compounds had no effect on NMDA-induced calcium accumulation.

Exposure to NMDA either alone or in the presence of other compounds was carried out for 5 minutes at room temperature in a HEPES-buffered salt solution (HBSS) containing 120 mM NaCl, 5.4 mM KCl, 0.8 mM MgCl2, 1.8 mM CaCl2, 20 mM HEPES, 15 mM glucose, and 0.01 mM glycine (pH 7.4). After 5 minutes, the exposure solution was washed away using three 750 µl washes and replaced by stock medium (2 mM glutamine and 20 mM glucose) supplemented with glycine (0.01 mM). Neuronal cell death was estimated by examination of cultures under phase-contrast microscopy and quantified by measurement of lactate dehydrogenase (LDH) released by damaged or destroyed cells into the bathing medium 1 day following experimentation. Data was expressed as a percentage of complete cell death, i.e., total LDH, which was determined in each experiment by assaying the supernatant of sister cultures exposed to 300 µM NMDA for 24 hours. Experiments were acceptable only if background LDH (determined from sham washed sister cultures) was less than 20% of the total LDH. In most cases, background LDH levels were subtracted from the levels in the experimental conditions to

yield the signal specific for experimental injury. To confirm the accuracy of LDH measurements, neuronal viability was measured using a spectrophotometric trypan blue absorbance assay. Mean absorbance values obtained from sham washed cultures were subtracted from all experimental conditions to yield absorbance values specific to experimental injury. Data were expressed as the percentage of absorbance measured from parallel cultures exposed for 20-24 hours to 300 μM NMDA which resulted in complete neuronal cell death (=100% trypan blue positive neurons).

9. I also examined the effect of compounds of the invention as inhibitors of stress-induced programmed neuronal cell death. After a 5 minute exposure of neurons to NMDA, the cells were incubated for 60 minutes, and prostaglandin production then assessed. ON 09250, ON 26040, and ON 09310 prevented oxidative stress-induced programmed neuronal cell death, as illustrated in attached Exhibit 10. Cell injury and cell death were assayed as described in section 8.

10. Additionally, I demonstrated that compounds of the present invention directly inhibit COX-2 cyclooxygenase activity in an in vitro enzymatic assay. Attached Exhibit 11 illustrates that ON 09250A and ON 09250B (which are enantiomers of the racemic compound ON 09250), and ON 09310A all inhibit the cyclooxygenase activity of COX-2 more effectively than does celecoxib. Cyclooxygenase activity of ovine COX-1 and COX-2 was assayed using a COX inhibitory screening assay kit (Cayman Chemicals, MI). This assay directly measures PGF_{2a} produced by stannous chloride reduction of COX-derived $PGFH_2$, by enzyme immunoassay (EIA). All assays were conducted in duplicate and IC_{50} values are the average of duplicate determinations for each compound. In brief, for the inhibition assay, hematin reconstituted purified COX-1 and COX-2 enzymes (6 units) in a reaction buffer containing Tris HCl (0.1M, pH 8.0), 5mM EDTA, 2mM phenol, were pre-incubated at room temperature for 1 hour with inhibitor concentrations ranging from 0.001 μM to 100 μM in DMSO followed by the addition of arachidonic acid (100 mM) for 2 minutes at room temperature. Reactions were terminated by adding 50 μL of 1 M HCl followed by the addition of 100 μL of saturated

stannous chloride. The final formed product, $PGF_{2\alpha}$, was measured by EIA and IC_{50} values were determined following the instructions given in the kit manual.

11. I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

1/17/2001
(Date)

M. V. Ramana Reddy, Ph.D.

Exhibit 1

PubMed Abstracts

1: Prostate. 2000 Jan;42(1):73-8.

Over-expression of cyclooxygenase-2 in human prostate adenocarcinoma.

Gupta S, Srivastava M, Ahmad N, Bostwick DG, Mukhtar H.

Department of Dermatology, Case Western Reserve University Cleveland, Ohio 44106, USA.

Aberrant or increased expression of cyclooxygenase (COX)-2 has been implicated in the pathogenesis of many diseases including carcinogenesis. COX-2 has been shown to be over-expressed in some human cancers. Employing semi-quantitative reverse transcription-PCR, immunoblotting, and immunohistochemistry we assessed COX-2 expression in samples of pair-matched benign and cancer tissue obtained from the same prostate cancer patient. Mean levels of COX-2 mRNA were 3.4-fold higher in prostate cancer tissue (n = 12) compared with the paired benign tissue. The immunoblot analysis demonstrated that as compared to benign tissue COX-2 protein was over-expressed in 10 of 12 samples examined. Immunohistochemical analysis also verified COX-2 over-expression in cancer than in benign tissue. To our knowledge, this is the first in vivo study showing an over-expression of COX-2 in prostate cancer. These data suggest that COX-2 inhibitors may be useful for prevention or therapy of prostate cancer in humans. Copyright 2000 Wiley-Liss, Inc.

PMID: 10579801 [PubMed - indexed for MEDLINE]

1: Carcinogenesis. 2000 Feb;21(2):139-46.

Cyclooxygenase-2 expression in human pancreatic adenocarcinomas.

Yip-Schneider MT, Barnard DS, Billings SD, Cheng L, Heilman DK, Lin A, Marshall SJ, Crowell PL, Marshall MS, Sweeney CJ.

Department of Medicine, Department of Biochemistry and Walther Oncology Center, Indiana University School of Medicine, Indianapolis 46202, USA. myipschn@iupui.edu

Cyclooxygenase-2 (COX-2) expression is up-regulated in several types of human cancers and has also been directly linked to carcinogenesis. To investigate the role of COX-2 in pancreatic cancer, we evaluated COX-2 protein expression in primary human pancreatic adenocarcinomas (n = 23) and matched normal adjacent tissue (n = 11) by immunoblot analysis. COX-2 expression was found to be significantly elevated in the pancreatic tumor specimens compared with normal pancreatic tissue. To examine whether the elevated levels of COX-2 protein observed in pancreatic tumors correlated with the presence of oncogenic K-ras, we determined the K-ras mutation status in a subset of the tumors and corresponding normal tissues. The presence of oncogenic K-ras did not correlate with the level of COX-2 protein expressed in the pancreatic adenocarcinomas analyzed. These observations were also confirmed in a panel of human pancreatic tumor cell lines. Furthermore, in the pancreatic tumor cell line expressing the highest level of COX-2 (BxPC-3), COX-2 expression was demonstrated to be independent of Erk1/2 activation. The lack of correlation between COX-2 and oncogenic K-ras expression suggests that Ras activation may not be sufficient to induce COX-2 expression in pancreatic tumor cells and that the aberrant activation of signaling pathways other than Ras may be required for up-regulating COX-2 expression. We also report that the COX inhibitors sulindac, indomethacin and NS-398 inhibit cell growth in both COX-2-positive (BxPC-3) and COX-2-negative (PaCa-2) pancreatic tumor cell lines. However, suppression of cell growth by indomethacin and NS-398 was significantly greater in the BxPC-3 cell line compared with the PaCa-2 cell line ($P = 0.004$ and $P < 0.001$, respectively). In addition, the three COX

inhibitors reduce prostaglandin E(2) levels in the BxPC-3 cell line. Taken together, our data suggest that COX-2 may play an important role in pancreatic tumorigenesis and therefore be a promising chemotherapeutic target for the treatment of pancreatic cancer.

PMID: 10657949 [PubMed - indexed for MEDLINE]

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Jun 27 2005 04:57:20

All: 1 Review: 0

1: Jpn J Cancer Res. 1999 Dec;90(12):1338-43.

Related Articles, Links

Japanese Cancer Association Cancer Science

Cyclooxygenase-2 (COX-2) mRNA expression levels in normal lung tissues and non-small cell lung cancers.

Ochiai M, Oguri T, Isobe T, Ishioka S, Yamakido M.

Second Department of Internal Medicine, Hiroshima University Faculty of Medicine, Kasumi.

One of the cyclooxygenase (COX) isoforms, COX-2, is overexpressed in various human cancers. In this study, we examined the gene expression levels of COX-2 in primary non-small cell lung cancers (NSCLC), metastatic lymph nodes, and normal lung tissues. The expression levels of the COX-2 gene were assessed by means of the reverse transcription polymerase chain reaction in 76 autopsy samples (29 primary NSCLC, 29 corresponding normal lung tissues, and 9 metastatic lymph nodes). The expression levels in NSCLC (both adenocarcinomas and squamous cell carcinomas) were significantly higher than in normal lung tissues and were significantly higher in adenocarcinomas than in squamous cell carcinomas. Differences between the levels of expression of COX-2 in primary tumors and their corresponding metastatic lymph nodes in 9 adenocarcinoma patients were not significant. Our results indicate that COX-2 may be associated with carcinogenesis of NSCLC, and that it may be a target for the treatment of NSCLC.

PMID: 10665651 [PubMed - indexed for MEDLINE]

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1: Am J Pathol. 2000 Jul;157(1):29-35.

Related Articles, Links

Enhanced expression of cyclooxygenase-2 in high grade human transitional cell bladder carcinomas.

Komhoff M, Guan Y, Shappell HW, Davis L, Jack G, Shyr Y, Koch MO, Shappell SB, Breyer MD.

Division of Nephrology, Department of Medicine, Vanderbilt University, Nashville, TN, USA.

Studies in human and animal models have shown that cyclooxygenase (COX)-2 is up-regulated in several epithelial carcinomas including colon, breast, and lung. To elucidate the possible involvement of COX-2 in human bladder cancer we examined the expression of COX isoforms in benign tissue and in bladder carcinoma specimens. Paraffin embedded tissues from 75 patients with urothelial carcinomas were immunostained with specific antibodies raised against COX-1 and COX-2. COX-1 expression was detected in smooth muscle cells in both benign and malignant bladders. COX-2 immunoreactivity was absent in benign tissue and in specimens with low-grade urothelial carcinoma (0/23). In contrast, expression of COX-2 was detected in malignant epithelial cells in 38% (17/47) of specimens with high-grade urothelial carcinomas. Expression of COX-2 in high-grade bladder cancer was confirmed by radioactive in situ hybridization using a COX-2-selective riboprobe. Both immunohistochemistry and in situ hybridization showed COX-2 expression in a small subset of malignant cells. COX-2 mRNA was also expressed in three out of seven malignant urothelial cell lines. These data demonstrate elevated expression of COX-2 in a high percentage of high-grade bladder carcinomas, suggesting a possible role of COX-2 in the progression of bladder urothelial carcinoma and supporting its potential as a therapeutic target in human bladder carcinoma.

PMID: 10880372 [PubMed - indexed for MEDLINE]

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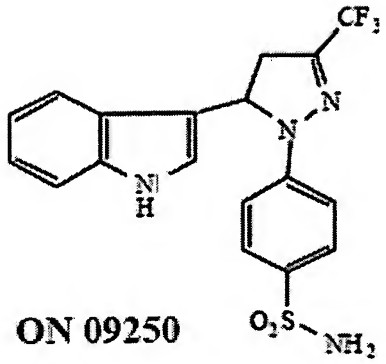
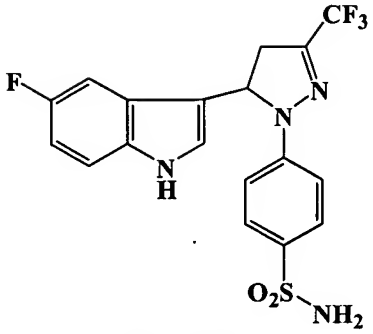
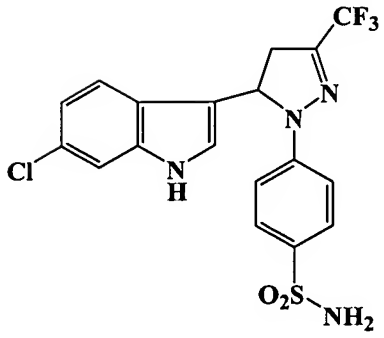
Exhibit 2



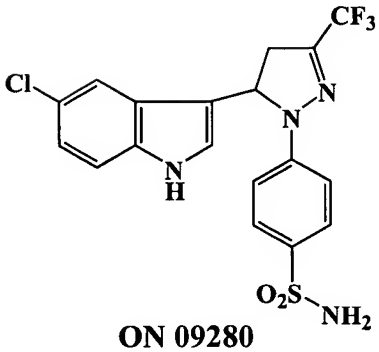
ON 09250



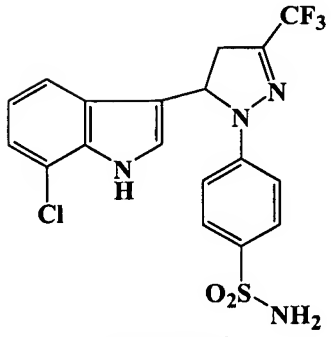
ON 09270



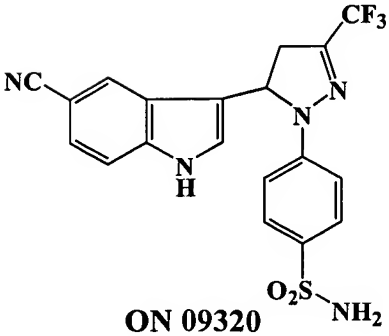
ON 09300



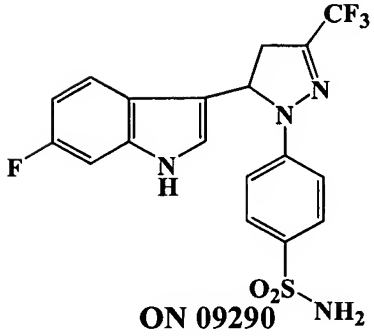
ON 09280



ON 09310



ON 09320



ON 09290

Exhibit 3

Soft Agar Assay (DLD-1 Cells)

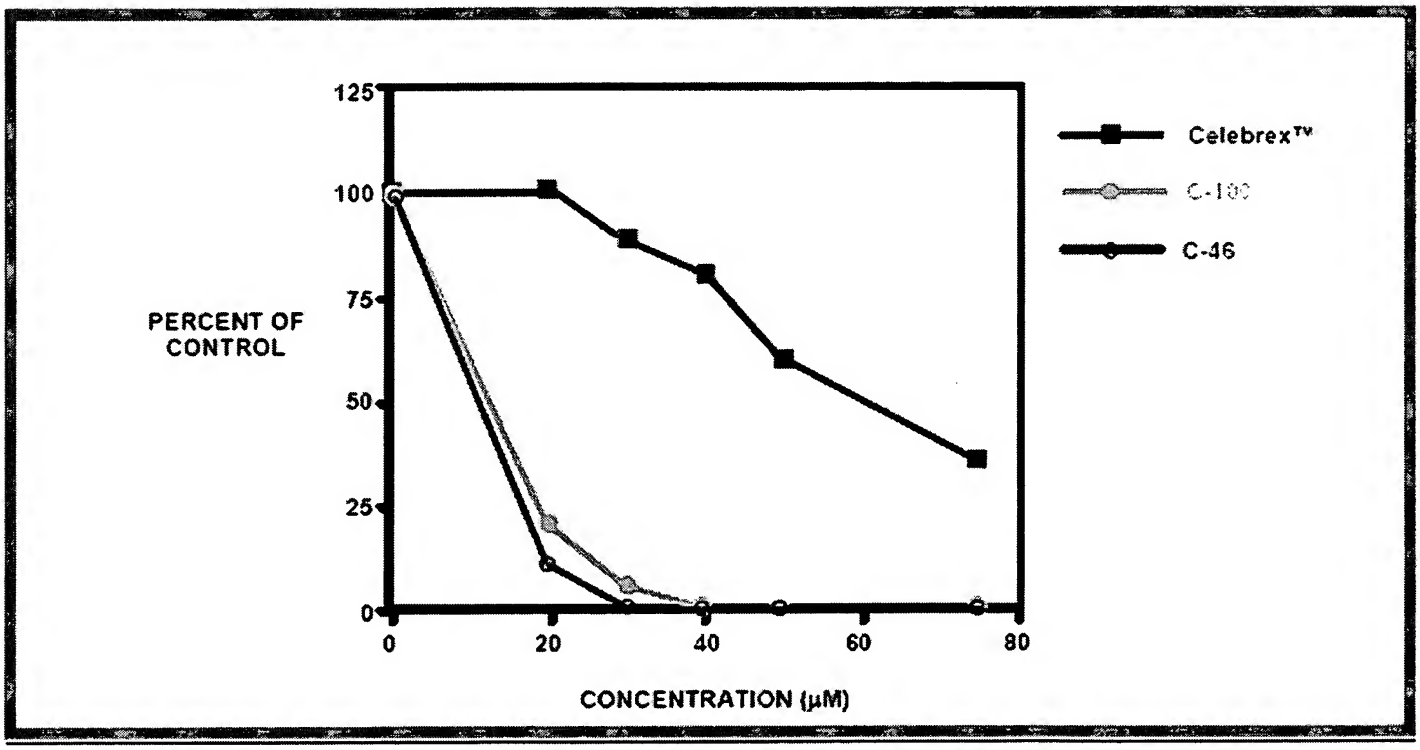


Exhibit 4

Soft Agar: HT-29 Cells Treated with ON 09250 and Celecoxib

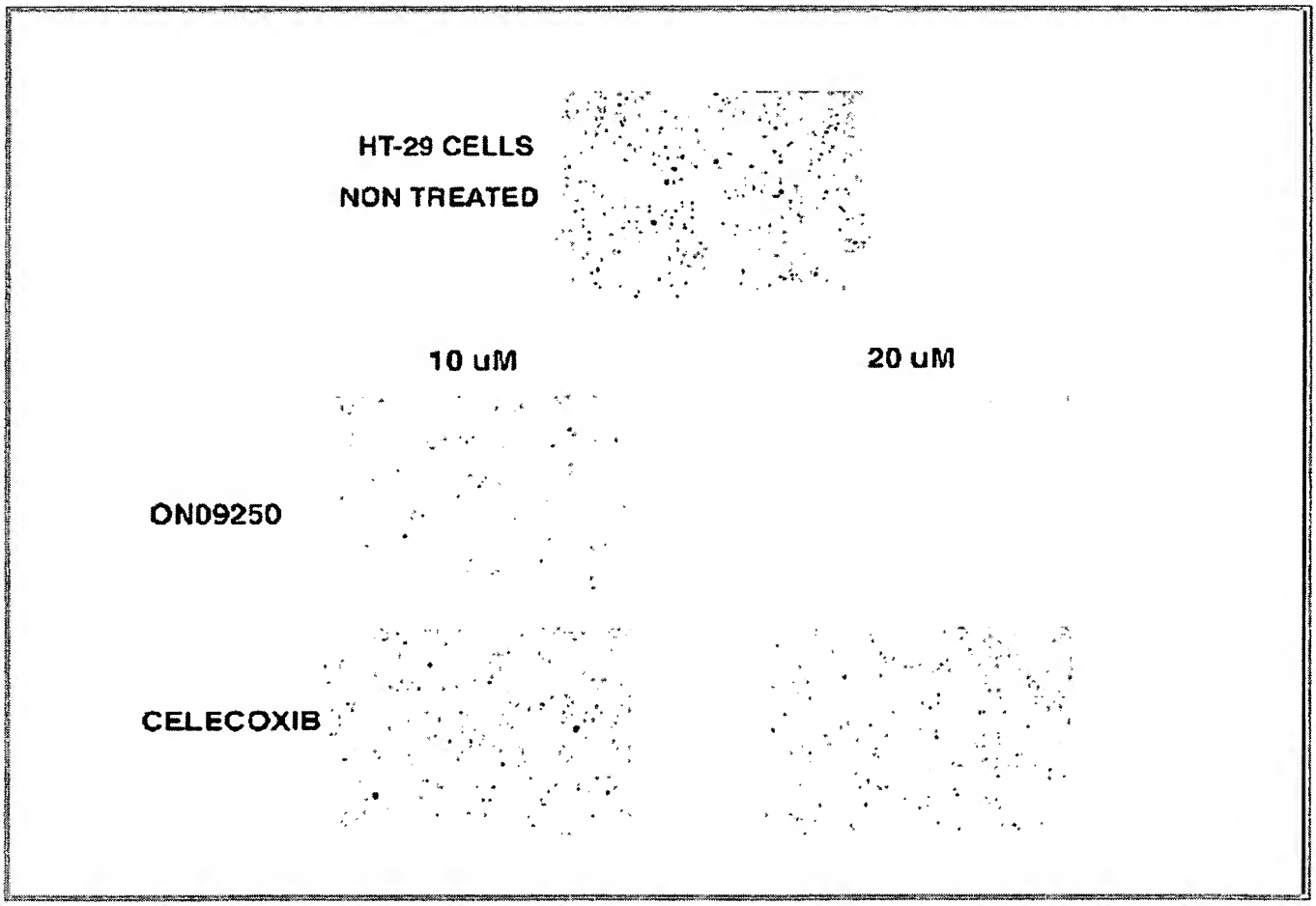


Exhibit 5

Dose Response of COX-2 Inhibitors: Colon Cancer (HT29) Cells

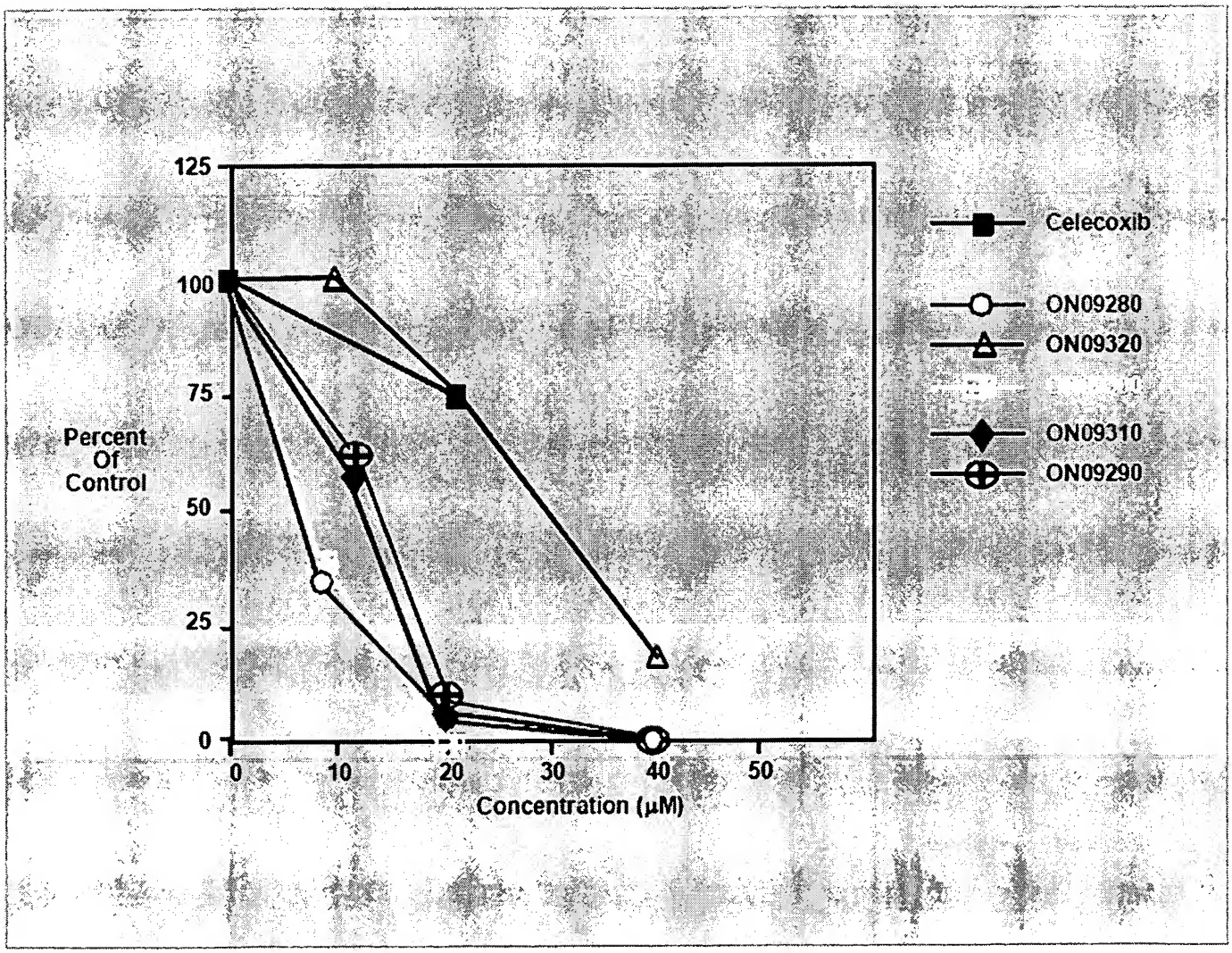
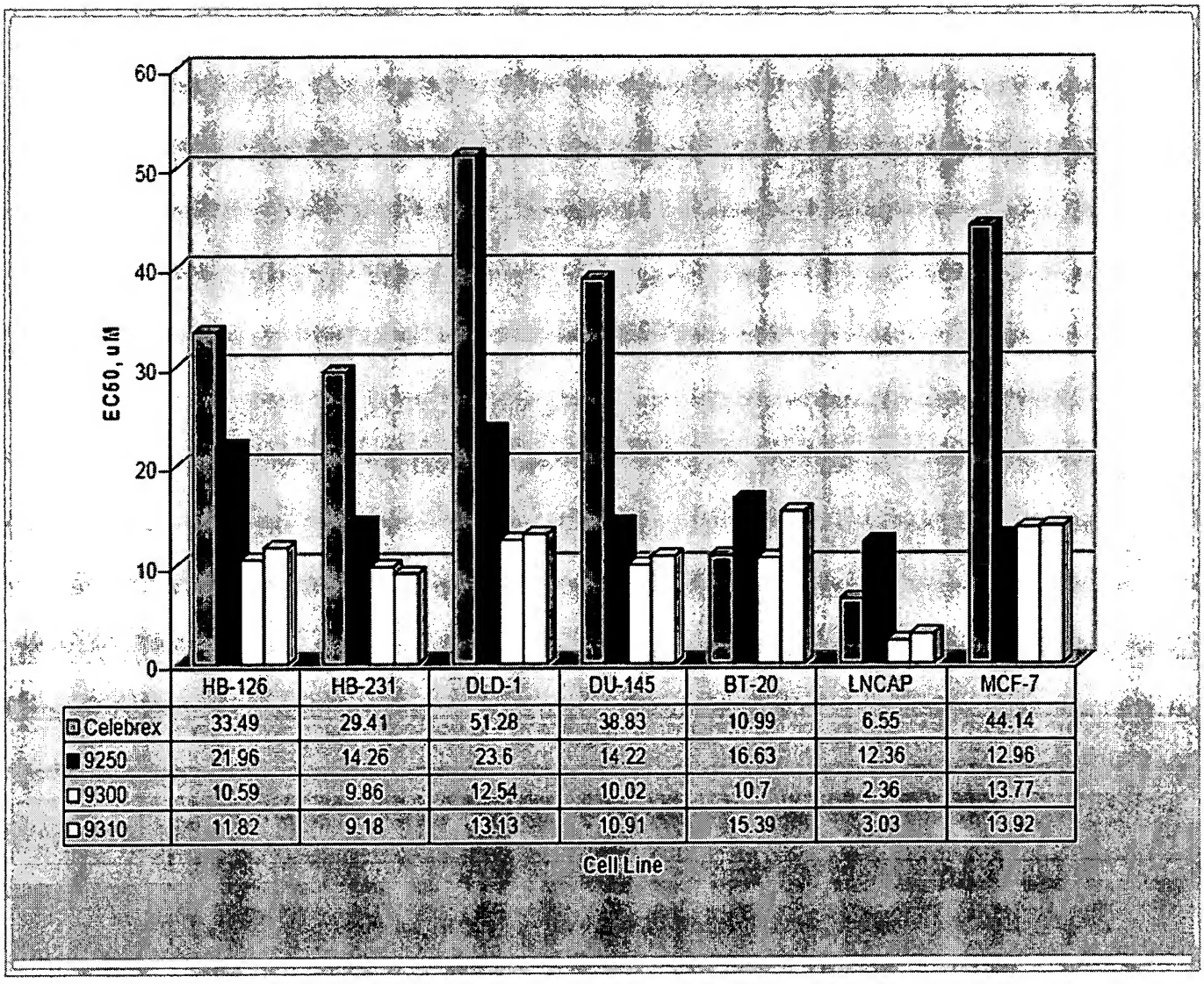


Exhibit 6

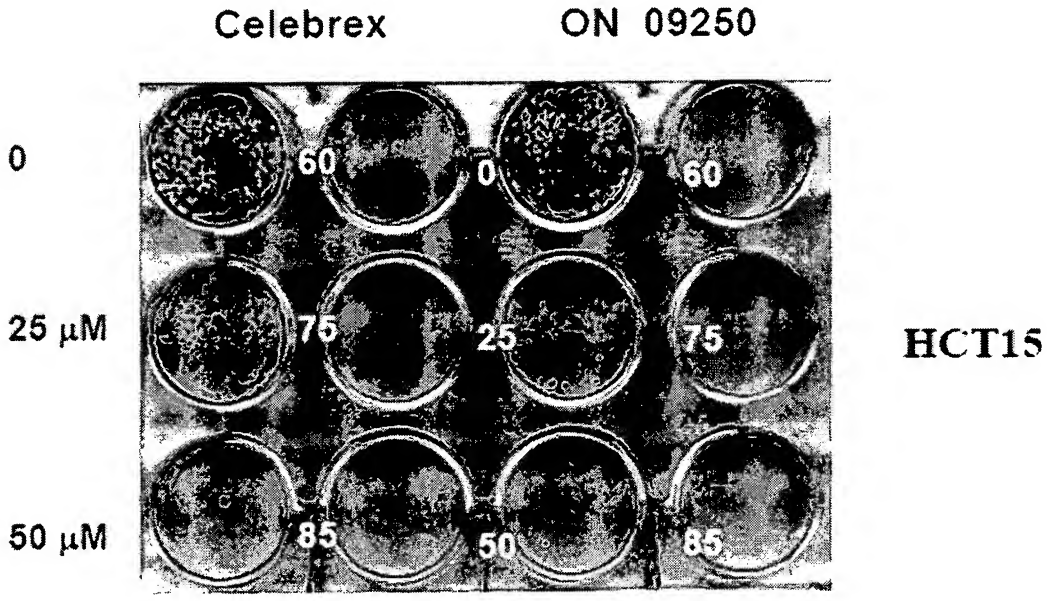
Effect of Cox-2 Inhibitors on Human Cell



	HB-126	HB-231	DLD-1	DU-145	BT-20	LNCAP	MCF-7
Celebrex	33.49	29.41	51.28	38.83	10.99	6.55	44.14
9250	21.96	14.26	23.6	14.22	16.63	12.36	12.96
9300	10.59	9.86	12.54	10.02	10.7	2.36	13.77
9310	11.82	9.18	13.13	10.91	15.39	3.03	13.92

Exhibit 7

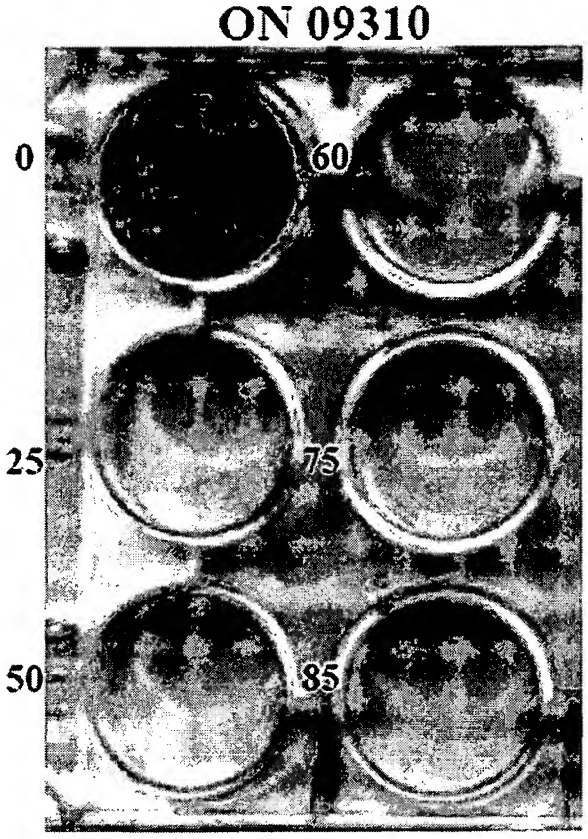
COMPARISON OF CELECOXIB AND ON 09250: COLON CANCER



HCT 15 Colon Cancer Cells Expressing COX-2

Exhibit 8

Potent Inhibitor of Colon Cancer Cell Growth



HCT15

Concentration used: 25 µM to 85 µM
Time: 96 h

Exhibit 9

Induction of Apoptosis in Human Colon and Breast Cancer Cells

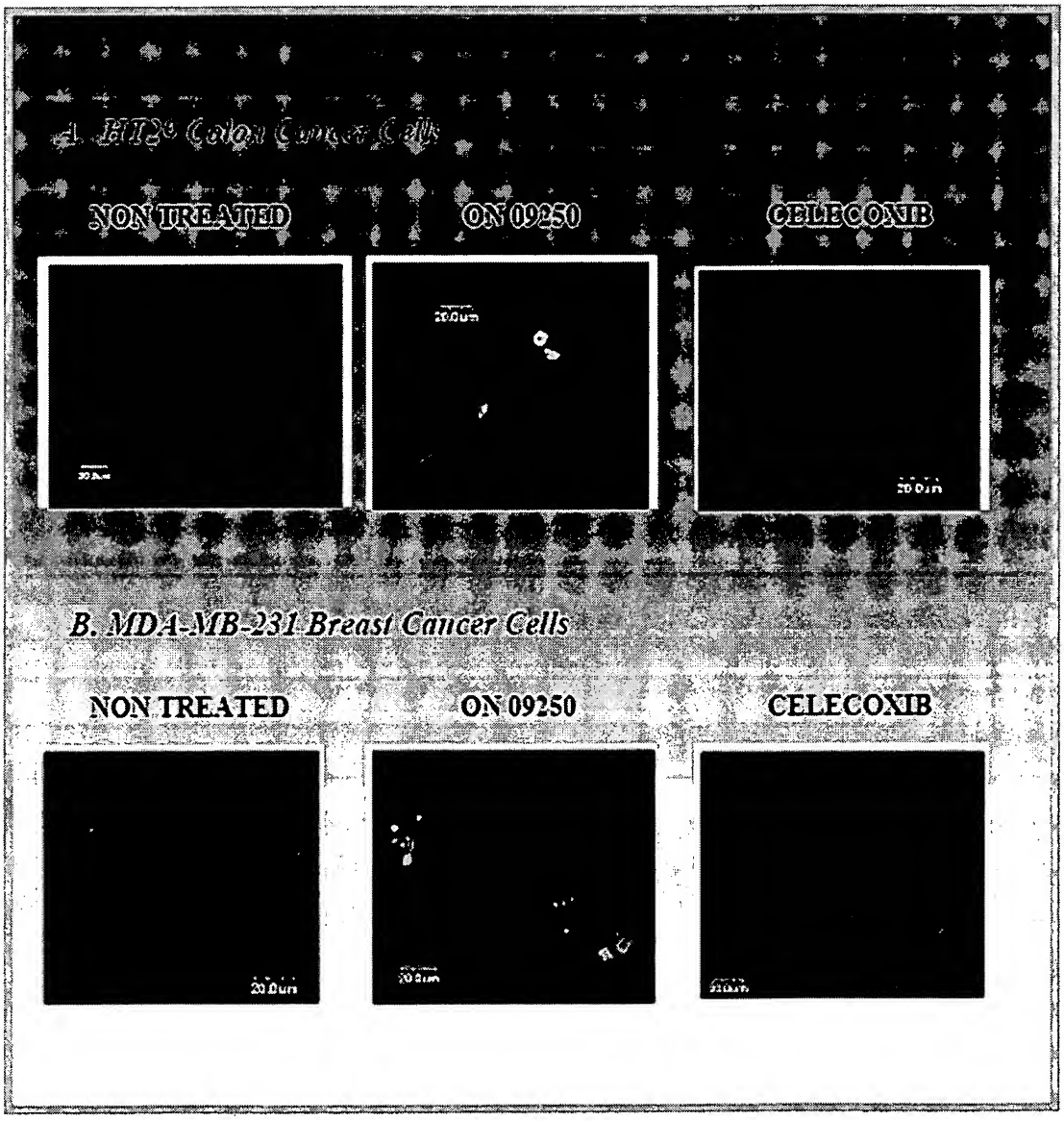
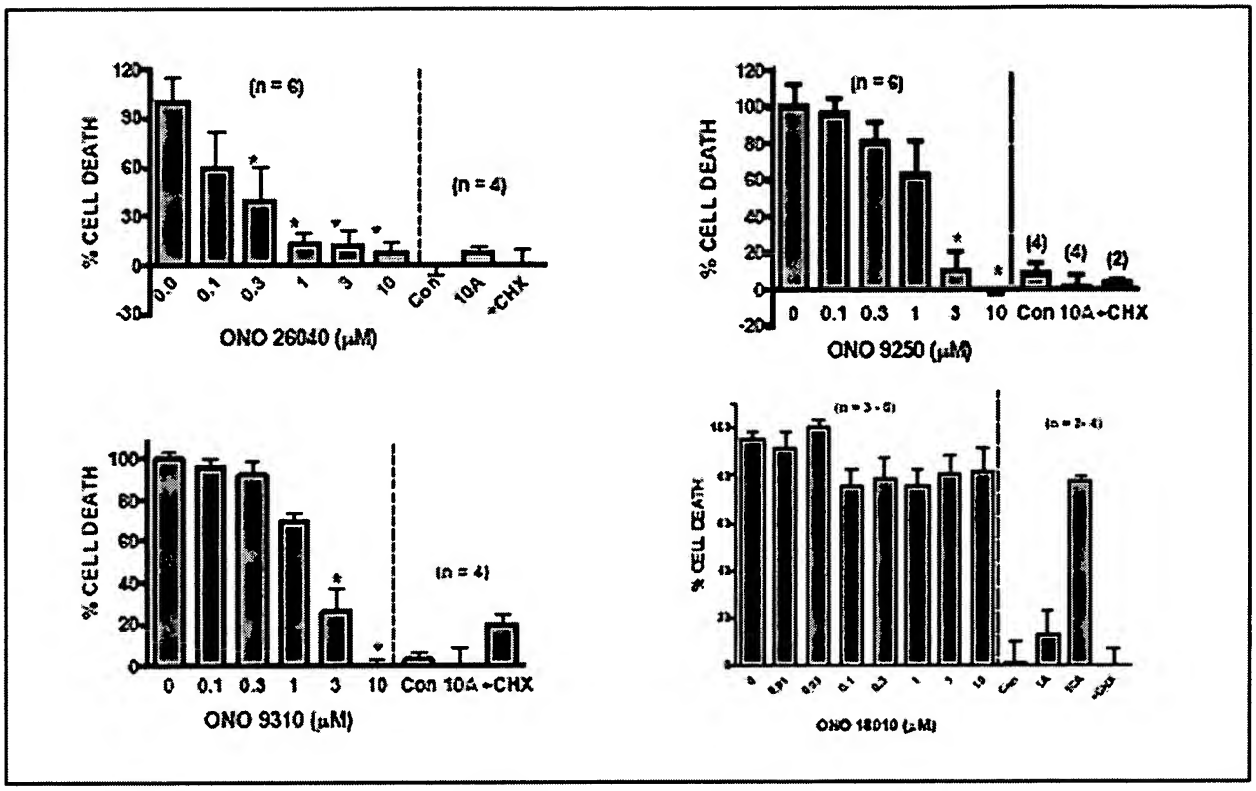
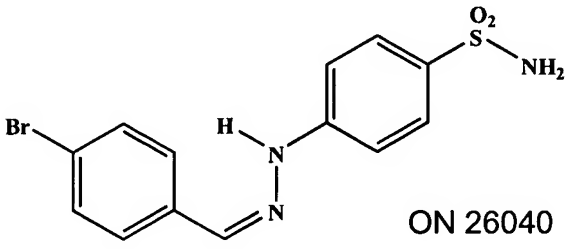


Exhibit 10

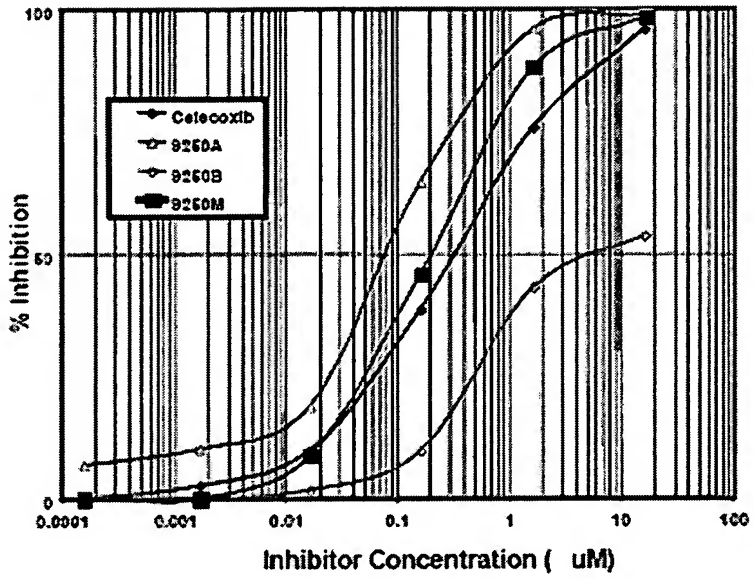


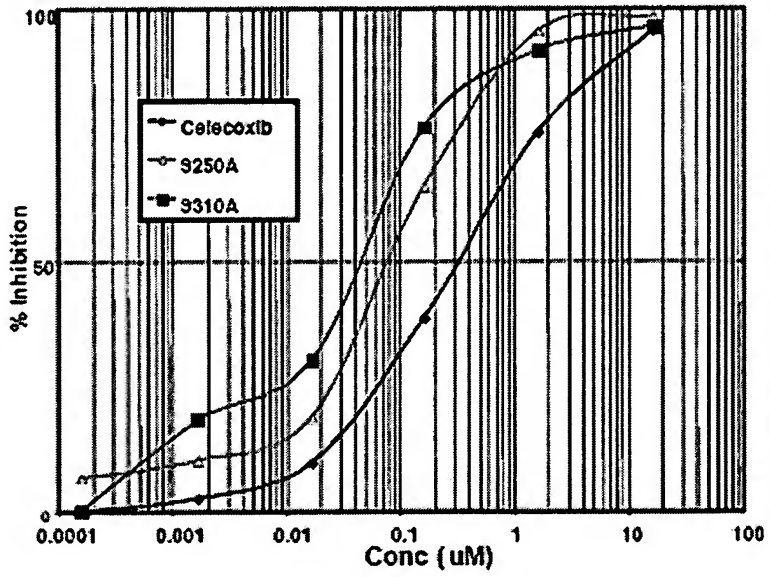
[Con = medium alone; 10A = 10μM drug alone; +CHX = HCA + cycloheximide]



ON 26040

Exhibit 11


100
50
0
% Inhibition
Celecoxib
9250A
9250B
9250M
0.0001
0.001
0.01
0.1
1
10
100
Inhibitor Concentration (uM)


100
50
0
% Inhibition
Celecoxib
9250A
9310A
0.0001
0.001
0.01
0.1
1
10
100
Conc (uM)